(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 November 2002 (21.11.2002)

PCT

(10) International Publication Number
WO 02/091969 A1

(51) International Patent Classification[7]: A61F 13/00

(21) International Application Number: PCT/US02/15394

(22) International Filing Date: 16 May 2002 (16.05.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/291,326 17 May 2001 (17.05.2001) US

(71) Applicant *(for all designated States except US)*: **REGENERX BIOPHARMACEUTICALS, INC.** [US/US]; 3 Bethesda Metro Center, Bethesda, MD 20814 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GOLDSTEIN, Allan, L.** [US/US]; 800 25th Street, N.W., Apartment 1005, Washington DC 20037 (US). **FINKELSTEIN, Jack, Jr.** [US/US]; 3703 Taylor Street, Chevy Chase, MD 20815 (US).

(74) Agents: **REPPER, George, R.** et al.; Rothwell, Figg, Ernst & Manbeck, P.C., Suite 800, 1425 K. Street, N.W., Washington, DC 20005 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** TREATING EPIDERMLYOSIS BULLOSA WITH THYMOSIN BETA 4

(57) **Abstract:** Blister, sores or skin degradation associated with Epidermolysis Bullosa is treated or prevented by administration of a actin-sequestering peptide such as Thymosin β4, an isoform of Thymosin β4 or oxidized Thymosin β4.

WO 02/091969 A1

"TREATING EPIDERMLYOSIS BULLOSA WITH THYMOSIN BETA 4"

BACKGROUND OF THE INVENTION

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit of U.S. Provisional Application Serial No. 60/291,326, filed May 17, 2001.

1. FIELD OF THE INVENTION

The present invention relates to the field of healing or preventing inflammatory degenerative, immunological and other disorders of the skin and surrounding tissue that occur due to Epidermolysis Bullosa, and all of its subtypes.

2. DESCRIPTION OF THE BACKGROUND ART

The phenomenon called Epidermolysis Bullosa (EB) is a rare genetic disorder that afflicts all ethnic and racial groups. EB is a group of diseases characterized by blister formation after minor trauma to the skin. This may lead to open sores, ulcerations and scars. This family of disorders range in severity from mild to the severely disabling, mutilating and life threatening diseases of the skin. Unlike burns, these afflictions sometimes never go away. The most severe cases require great lifestyle adjustments. Afflicted children should never ride a bike, skate, or participate in sports, because the normal play of children causes chronic sores. Such sores may cover as much as 75 percent of the child's body. Blistering and scarring also occur in the mouth and esophagus. Therefore, frequently a diet of only liquids or soft foods is possible. Scarring also causes the fingers and toes to fuse, leaving deformities which severely limit function. Much of their life is tied to hospitals for treatment, blood transfusions, biopsies and surgeries. The eyes often blister preventing sight for days. Chronic anemia reduces energy, and growth is retarded. The life span for an individual afflicted with EB is usually not longer than 30 years.

There are three main types of EB: EB Simplex, Dystrophic EB (dominant or recessive) and Junctional EB. The severity of symptoms varies between these types. In general terms, EB causes blisters which may be restricted to specific areas, for example hands or feet, or may affect large areas of the body. In the milder forms the blisters heal normally without leaving permanent damage to the skin. In the other forms, the blisters

heal with scarring which can result in permanent change to the skin, for example fingers may fuse and hands contract, reducing movement. Some forms of Junctional EB are life threatening in infancy.

EB results from deleterious changes in the physiological, biochemical and immunological properties of the skin. All forms of EB are genetic in origin and the genes responsible for several different subtypes of the condition are now known. The genetic defects result in the skin layers not adhering properly to each other, causing areas of structural weakness. This fragile skin is particularly vulnerable to damage from mild friction, causing the blisters which are the characteristic feature of the condition. Skin is an important barrier to infection, it is the first line of defense of the immune system. The fragile skin of those afflicted with EB loses this important defense mechanism. Such changes in vasculature decrease capacity to repair damage, increase propensity for skin cancers such as squamous cell carcinoma, and increase risk of infection. In addition, the open sores in the oral and digestive cavity can lead to increased dehydration and malnutrition.

There have been many attempts to treat EB, but none have had a substantial impact on prevention or treatment of EB. Various growth factors, synthetic skins, antibiotics and other therapies have failed to adequately and effectively treat EB. While EB is a genetic disease, treatment that would more rapidly heal or better heal the sores would be extremely important. Further, preventative therapy would clearly confer substantial, perhaps life-saving benefit to the patient.

Numerous pharmaceutical, nutriceutical or cosmeceutical formulations have been proposed to reduce or reverse EB or its affects.

There remains a need in the art for improved methods and compositions for healing or preventing the blisters and sores associated with EB.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method of treatment for promoting healing or prevention of blisters, sores or skin degeneration associated with EB involves administration to a subject or patient in need of such treatment an effective amount of a composition comprising an EB-inhibiting polypeptide comprising amino acid sequence LKKTET or a conservative variant thereof having EB-inhibiting activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on a discovery that actin-sequestering peptides such as thymosin β4 (Tβ4) and other actin-sequestering peptides or peptide fragments containing amino acid sequence LKKTET or conservative variants thereof, promote healing or prevention of blisters, sores and skin degeneration associated with Epidermolysis Bullosa. Included are N– or C-terminal variants such as KLKKTET and LKKTETQ. Without being bound to any particular theory, these peptides may have the capacity to promote repair, healing and prevention by having the ability to induce terminal deoxynucleotidyl transferase (a non-template directed DNA polymerase), to decrease the levels of one or more inflammatory cytokines or chemokines, and to act as a chemotactic and/or angiogenic factor for endothelial cells and thus heal and prevent degenerative changes in the skin of patients with afflicted EB, even though EB is the result of an inherited defect.

Thymosin β4 was initially identified as a protein that is up-regulated during endothelial cell migration and differentiation *in vitro*. Thymosin β4 was originally isolated from the thymus and is a 43 amino acid, 4.9 kDa ubiquitous polypeptide identified in a variety of tissues. Several roles have been ascribed to this protein including a role in a endothelial cell differentiation and migration, T cell differentiation, actin sequestration and vascularization.

In accordance with one embodiment, the invention is a method of treatment for promoting healing and prevention of blisters, sores and skin degradation associated with EB comprising administering to a subject in need of such treatment an effective amount of a composition comprising an EB-inhibiting peptide comprising amino acid sequence LKKTET, or a conservative variant thereof having EB-inhibiting activity, preferably Thymosin β4, an isoform of Thymosin β4, oxidized Thymosin β4, Thymosin β4 sulfoxide, or an antagonist of Thymosin β4.

Compositions which may be used in accordance with the present invention include Thymosin β4 (Tβ4), Tβ4 isoforms, oxidized Tβ4, Thymosin β4 sulfoxide, polypeptides or any other actin sequestering or bundling proteins having actin binding domains, or peptide fragments comprising or consisting essentially of the amino acid sequence LKKTET or conservative variants thereof, having EB-inhibiting activity. International Application Serial No. PCT/US99/17282, incorporated herein by reference, discloses isoforms of Tβ4 which may be useful in accordance with the present invention as well as amino acid sequence LKKTET and conservative variants thereof having EB-inhibiting activity, which may be utilized with the present invention. International Application Serial

No. PCT/GB99/00833 (WO 99/49883), incorporated herein by reference, discloses oxidized Thymosin β4 which may be utilized in accordance with the present invention. Although the present invention is described primarily hereinafter with respect to Tβ4 and Tβ4 isoforms, it is to be understood that the following description is intended to be equally applicable to amino acid sequence LKKTET, peptides and fragments comprising or consisting essentially of LKKTET, conservative variants thereof having EB-inhibiting activity, as well as oxidized Thymosin β4.

In one embodiment, the invention provides a method for healing and preventing blisters and sores of skin in a subject by contacting the skin with an EB-inhibiting effective amount of a composition which contains Tβ4 or a Tβ4 isoform. The contacting may be topically or systemically. Examples of topical administration include, for example, contacting the skin with a lotion, salve, gel, cream, paste, spray, suspension, dispersion, hydrogel, ointment, or oil comprising Tβ4, alone or in combination with at least one agent that enhances Tβ4 penetration, or delays or slows release of Tβ4 peptides into the area to be treated. Systemic administration includes, for example, intravenous, intraperitoneal, intramuscular or subcutaneous injections, or inhalation, transdermal or oral administration of a composition containing Tβ4 or a Tβ4 isoform, etc. A subject may be a mammal, preferably human.

Tβ4, or its analogues, isoforms or derivatives, may be administered in any suitable EB-inhibiting amount. For example, Tβ4 may be administered in dosages within the range of about 0.1-50 micrograms of Tβ4, more preferably in amounts of about 1-25 micrograms.

A composition in accordance with the present invention can be administered daily, every other day, etc., with a single administration or multiple administrations per day of administration, such as applications 2, 3, 4 or more times per day of administration.

Tβ4 isoforms have been identified and have about 70%, or about 75%, or about 80% or more homology to the known amino acid sequence of Tβ4. Such isoforms include, for example, $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15. Similar to Tβ4, the Tβ10 and Tβ15 isoforms have been shown to sequester actin. Tβ4, Tβ10 and Tβ15, as well as these other isoforms share an amino acid sequence, LKKTET, that appears to be involved in mediating actin sequestration or binding. Although not wishing to be bound to any particular theory, the activity of Tβ4 isoforms may be due, in part, to the ability to regulate the polymerization of actin. For example, Tβ4 can modulate actin polymerization in skin (*e.g.* β-thymosins appear to depolymerize F-actin by sequestering free G-actin). Tβ4's ability to modulate actin polymerization may therefore be due to all,

or in part, its ability to bind to or sequester actin via the LKKTET sequence. Thus, as with Tβ4, other proteins which bind or sequester actin, or modulate actin polymerization, including Tβ4 isoforms having the amino acid sequence LKKTET, are likely to reduce EB, alone or in a combination with Tβ4, as set forth herein.

Thus, it is specifically contemplated that known Tβ4 isoforms, such as $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15, as well as Tβ4 isoforms not yet identified, will be useful in the methods of the invention. As such Tβ4 isoforms are useful in the methods of the invention, including the methods practiced in a subject. The invention therefore further provides pharmaceutical compositions comprising Tβ4, as well as Tβ4 isoforms $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15, and a pharmaceutically acceptable carrier.

In addition, other proteins having actin sequestering or binding capability, or that can mobilize actin or modulate actin polymerization, as demonstrated in an appropriate sequestering, binding, mobilization or polymerization assay, or identified by the presence of an amino acid sequence that mediates actin binding, such as LKKTET, for example, can similarly be employed in the methods of the invention. Such proteins include gelsolin, vitamin D binding protein (DBP), profilin, cofilin, adsevertin, propomyosin, fincilin, depactin, DnaseI, vilin, fragmin, severin, capping protein, β-actinin and acumentin, for example. As such methods include those practiced in a subject, the invention further provides pharmaceutical compositions comprising gelsolin, vitamin D binding protein (DBP), profilin, cofilin, depactin, DnaseI, vilin, fragmin, severin, capping protein, β-actinin and acumentin as set forth herein. Thus, the invention includes the use of an EB-inhibiting polypeptide comprising the amino acid sequence LKKTET (which may be within its primary amino acid sequence) and conservative variants thereof.

As used herein, the term "conservative variant" or grammatical variations thereof denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the replacement of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

Tβ4 has been localized to a number of tissue and cell types and thus, agents which stimulate the production of Tβ4 can be added to or comprise a composition to effect Tβ4 production from a tissue and/or a cell. Such agents include members of the family of growth factors, such as insulin-like growth factor (IGF-1), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β),

basic fibroblast growth factor (bFGF), thymosin α1 (Tα1) and vascular endothelial growth factor (VEGF). More preferably, the agent is transforming growth factor beta (TGF-β) or other members of the TGF-β superfamily. Tβ4 compositions of the invention may reduce the affects of EB by effectuating growth of the connective tissue through extracellular matrix deposition, cellular migration and vascularization of the skin.

In accordance with one embodiment, subjects are treated with an agent that stimulates production in the subject of an EB-inhibiting peptide as defined above.

Additionally, agents that assist or stimulate EB reduction may be added to a composition along with Tβ4 or a Tβ4 isoform. Such agents include angiogenic agents, growth factors, agents that direct differentiation of cells, agents that promote migration of cells and agents that stimulate the provision of extracellular matrix material in the skin. For example, and not by way of limitation, Tβ4 or a Tβ4 isoform alone or in combination can be added in combination with any one or more of the following agents: VEGF, KGF, FGF, PDGF, TGFβ, IGF-1, IGF-2, IL-1, prothymosin α and thymosin α1 in an effective amount.

The invention also includes a pharmaceutical composition comprising a therapeutically effective amount of Tβ4 or a Tβ4 isoform in a pharmaceutically acceptable carrier. Such carriers include those listed above with reference to parenteral administration.

The actual dosage or reagent, formulation or composition that heals or prevents blisters, sores and skin degeneration associated with EB may depend on many factors, including the size and health of a subject. However, persons of ordinary skill in the art can use teachings describing the methods and techniques for determining clinical dosages as disclosed in PCT/US99/17282, *supra*, and the references cited therein, to determine the appropriate dosage to use.

Suitable topical formulations include Tβ4 or a Tβ4 isoform at a concentration within the range of about 0.001 - 10% by weight, more preferably within the range of about 0.01 - 0.1% by weight, most preferably about 0.05% by weight.

The therapeutic approaches described herein involve various routes of administration or delivery of reagents or compositions comprising the Tβ4 or other compounds of the invention, including any conventional administration techniques (for example, but not limited to, topical administration, local injection, inhalation, or systemic administration), to a subject. The methods and compositions using or containing Tβ4 or other compounds of the invention may be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers.

The invention includes use of antibodies which interact with Tβ4 peptide or functional fragments thereof. Antibodies which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art as disclosed in PCT/US99/17282, *supra*. The term antibody as used in this invention is meant to include monoclonal and polyclonal antibodies.

In yet another embodiment, the invention provides a method of treating a subject by administering an effective amount of an agent which modulates Tβ4 gene expression. The term "modulate" refers to inhibition or suppression of Tβ4 expression when Tβ4 is over expressed, and induction of expression when Tβ4 is under expressed. The term "effective amount" means that amount of Tβ4 agent which is effective in modulating Tβ4 gene expression resulting in reducing the symptoms of the Tβ4 associated EB. An agent which modulates Tβ4 or Tβ4 isoform gene expression may be a polynucleotide for example. The polynucleotide may be an antisense, a triplex agent, or a ribozyme. For example, an antisense directed to the structural gene region or to the promoter region of Tβ4 may be utilized.

In another embodiment, the invention provides a method for utilizing compounds that modulate Tβ4 activity. Compounds that affect Tβ4 activity (*e.g.*, antagonists and agonists) include peptides, peptidomimetics, polypeptides, chemical compounds, minerals such as zincs, and biological agents.

While not be bound to any particular theory, the present invention may promote healing or prevention of blisters, sores and skin degeneration associated with Epidermolysis Bullosa by inducing terminal deoxynucleotidyl transferase (a non-template directed DNA polymerase), to decrease the levels of one or more inflammatory cytokines, or chemokines, and to act as a chemotactic factor for endothelial cells, and thereby promoting healing or preventing degenerative changes in skin brought about by Epidermolysis Bullosa or other degenerative or environmental factors.

CLAIMS

1. A method of treatment for promoting healing or prevention of blisters, sores or skin degeneration associated with Epidermolysis Bullosa, comprising administering to a subject in need of such treatment an effective amount of a composition comprising an Epidermolysis Bullosa-inhibiting polypeptide comprising amino acid sequence LKKTET, or a conservative variant thereof having Epidermolysis Bullosa-inhibiting activity.

2. The method of claim 1 wherein said polypeptide promotes a skin condition improvement including an increase in skin elasticity of said subject.

3. The method of claim 1 wherein said polypeptide comprises Thymosin β4 (Tβ4), an N-terminal variant of Tβ4, a C-terminal variant of Tβ4, an isoform of Tβ4, oxidized Tβ4 or Tβ4 sulfoxide.

4. The method of claim 1 wherein said composition is administered systemically.

5. The method of claim 1 wherein said composition is administered topically.

6. The method of claim 5 wherein said composition is in the form of a gel, creme, paste, lotion, spray, suspension, dispersion, salve, hydrogel or ointment formulation.

7. The method of claim 6, further including at least one agent that delays release or enhances penetration of Tβ4 into an area to be treated.

8. The method of claim 1 wherein said polypeptide is recombinant or synthetic.

9. The method of claim 1 wherein said polypeptide is an antibody.

10. The method of claim 9 wherein said antibody is polyclonal or monoclonal.

11. A method of treatment for promoting healing or prevention of blisters, sores or skin degeneration associated with Epidermolysis Bullosa comprising administering to a subject in need of such treatment an effective amount of a composition comprising an agent that stimulates production of an Epidermolysis Bullosa-inhibiting polypeptide

comprising amino acid sequence LKKTET, or a conservative variant thereof having Epidermolysis Bullosa-inhibiting activity.

12. The method of claim 11 wherein said polypeptide is Thymosin β4.

13. The method of claim 11 wherein said agent is an antagonist of Thymosin β4.

14. A composition for use in promoting healing or prevention of blisters, sores or skin degeneration associated with Epidermolysis Bullosa comprising an effective amount of a composition including an Epidermolysis Bullosa-inhibiting polypeptide comprising amino acid sequence LKKTET or a conservative variant thereof having Epidermolysis Bullosa-inhibiting activity.

15. The composition of claim 14 wherein said composition comprises an N- or C-terminal variant of LKKTET.

16. The composition of claim 14 wherein said composition comprises KLKKTET or LKKTETQ.

17. The composition of claim 14 wherein said polypeptide comprises Tβ4, an isoform of Tβ4, oxidized Tβ4 or Tβ4 sulfoxide.

18. The composition of claim 14, comprising a gel, creme, paste, lotion, spray, suspension, dispersion salve, hydrogel or ointment formulation.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/15894

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61F 13/00
US CL :424/449, 443

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/449, 443

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST
SEARCH TERMS: topical, epidermolysis bullosa, thymosin.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,087,341 A (KHAVARI et al.) 11 July 2000, abstract; col. 6, lines 62-67; col. 7, lines 19-25, 44-48; col. 8, lines 59-69; col. 26, lines 51-53.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 31 JULY 2002	Date of mailing of the international search report 21 OCT 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JANE BUDGEON for SUSIS GHALI
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)★